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Refractive Index Determination of Biological Particles

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14. ABSTRACT

Refractive indices of biological particles have been determined using a new quantitative method based upon phase contrast immersion spectrometry. The new technique is sensitive, accurate, and requires only an optical transmission spectrophotometer and index matching fluids. The method was developed and tested using colloidal silica standard samples. Refractive indices for bacterial spores have been determined, and the results compared favorably with available data in the literature.

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Refractive Index Determination of Biological Particles

1. Introduction

The determination of colloidal refractive indices is important for a variety of reasons in a wide range of fields. Most notably, for inorganic and organic particle size determinations using light scattering, an estimate of refractive index must be given to calculate the particle size from the scattering data. In the biological realm, refractive index is important in terms of phase contrast microscopy[1, 2], flow cytometry, and light scatter based detection methods. With the recent requirements for substantially improved bio-warfare defense technologies, a better understanding of the optical properties of bio-agents, including refractive index, serves to enhance existing and emerging optical detection technologies.

The refractive index determination of liquids is well established. It can be performed using an Abbe or reflection type refractometer. Typically, in these types of instruments, light passing through (or reflecting from) an optical interface with the liquid in question is analyzed for angular displacement. The extent of displacement is related to the refractive index of the liquid. As light refraction occurs at the exact boundary between the two chemically different regions, it is a bulk sample measurement technique. Even in a concentrated colloidal sample, the dispersed colloid has no effect on the measured liquid refractive index.

Another important research area requiring knowledge of colloidal refractive index is optical trapping[3, 4, 5, 6]. This technique uses laser light to trap particles based upon photon momentum transfer when light is incident on colloidal samples. The direct non-contact manipulation afforded by optical trapping techniques can be applied to a wide range of particles including inorganic, organic, polymeric, metallic, and biological species. The ability to optically trap a particle depends upon their size, shape and refractive index. Until recently, the refractive index dependence has been known but largely neglected as a parameter for the optical discrimination of colloids. Optical chromatography, a technique related to optical trapping, has been used for the separation of particles based upon size[7, 8] and more recently, refractive index[9, 10]. Theoretical estimates of refractive index based separations indicate an exquisite sensitivity to differences in chemical composition (as measured by refractive index change)[9]. With the immerging possibilities in refractive index based separations (i.e. those based upon chemical differences between particles), refractive index determination is becoming increasingly important.

The technique demonstrated in this paper was borne out of the desire to develop a simplified and improved method for particle refractive index determination. In the techniques used in the past [11, 12], particles were observed to disappear when immersed in a surrounding medium whose refractive index matched that of the particles in question. In practice, this was done under a microscope for a limited number of individuals and the matching refractive index was signaled by the “disappearance” or “phase reversal” of the particles. This resulted in a fuzzy refractive index match point that inherently relied on a qualitative image judgment. Other more automated and reproducible instrumental techniques have been developed but are subject to calculation errors and/or involve costly instrumental apparatus.[13, 14]

In the current method, the microscope image is replaced by optical density measurements. The typically turbid colloidal suspension in water will become optically

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clear when the refractive index of the immersion liquid matches that of the suspended colloids. This method is simple and can be performed by measuring the optical transmission using only a basic spectrophotometer and index matching liquids. Although a few researchers tried previously to use this technique to determine the refractive indices of bacterial cells and spores [14,17,18] this is the first such a comprehensive description of this method.

2. Method

Using immersion liquids in conjunction with a spectrophotometer (Cary Bio 100, Varian, Inc.), the refractive indices of the samples can easily be determined through the measurement of optical density. A series of immersion liquids with increasing refractive index are created by either dissolving the appropriate amount of material or by mixing two standard refractive index liquids. The refractive indices of the immersion solutions were measured using a refractometer (Lecia AR200, Leica Microsystems, Inc., Buffalo, New York). The refractometer uses a light source with a wavelength of 589 nm, so this wavelength is also chosen for measuring the optical transmission. Standard quantities of particles to be analyzed are then introduced into each of the immersion liquids. The result is a series of solutions with equal colloidal concentrations but in liquids of varying refractive index. As the refractive index of the immersion solution becomes close to that of the immersed particles, the optical density approaches zero (100 % light transmission). The goal is to match, as closely as possible, the refractive index of the particles and solution and thus achieve maximum optical transmission. This often requires an iterative approach where additional solutions are prepared at higher or lower refractive indices to better match the suspended colloids.

Glass and plastic cuvettes were evaluated with respect to similarity as the use of matched pairs was deemed difficult given the number of samples required for the accurate determination of refractive index. Further, the use of plastic cuvettes with tapered reservoirs would reduce the volume of immersion liquid needed for analysis. The results given in Figure 1 show that the plastic cuvettes exhibit substantially less deviation than do the glass cuvettes. The glass cuvettes had a mean transmission of 99.5 % ± 0.4 % versus a mean transmission of 100.1 % ± 0.1 % for the plastic cuvettes (a 4-fold improvement in the standard deviation).

For the 2.3 μm diameter silica particles (Bangs Laboratories, Inc., Fishers, IN), immersion mixtures were water solutions of Triton X-100 of increasing concentration of Triton supplemented with 20% of ethanol to lower the viscosity of the resulting liquids. The refractive indices of these immersion liquids ranged from n = 1.3700 to n =1.4600. Standard commercially available organic refractive index liquids were used for immersion experiments to determine the refractive index of spores. Mixtures were prepared from standard liquids with refractive indices of n = 1.458 and n =1.570 (Series 5040, Cargille Laboratories, Inc.). The difficulty was that the spores were immersed in aqueous solutions incompatible with the organic immersion liquids. Due to the tough and resilient nature of bacterial spores, a method to transfer them into the organic medium without damage was devised. The spores were rinsed with isopropanol to make them compatible with the organic immersion liquid.

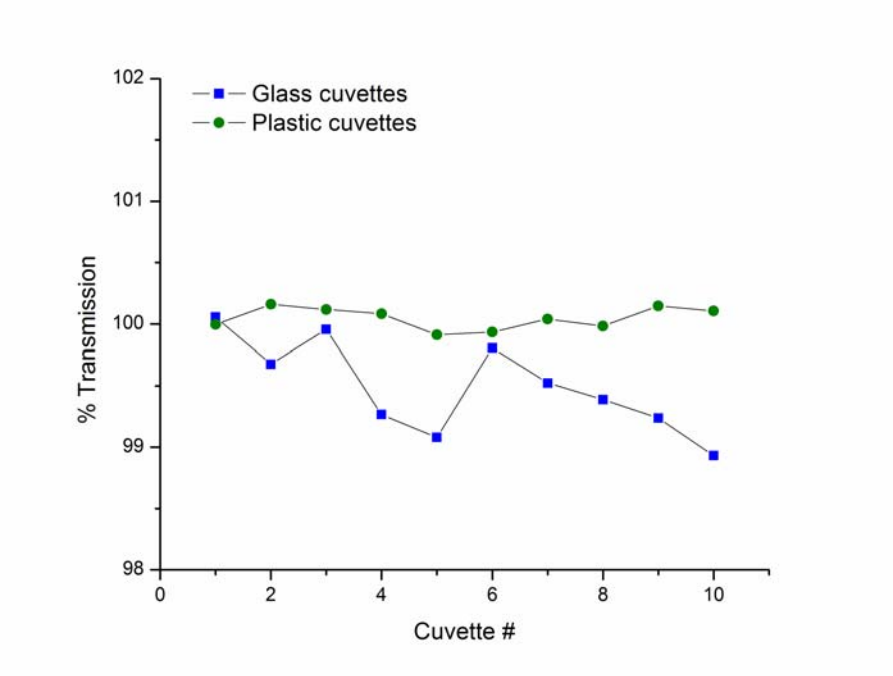

Figure 1. Comparison of optical transmission of water for ten glass and plastic cuvettes

They were first suspended in isopropanol, then resuspended, followed by centrifugation for 2 minutes to form a spore pellet and the process was repeated in triplicate. The samples were then prepared as follows: 40 µL aliquot of the resulting isopropanol/spore solution or 10 µL of isopropanol silica suspensions was added to 0.8 mL of each immersion liquid of differing refractive index. The refractive properties of the spores did not appear to change even after considerable time left in the immersion liquids (same refractive index results obtained after many weeks stored in immersion liquids, data not shown).

The following *Bacillus* strains were used in this study: *Bacillus anthracis* Sterne strain 34F2 (nonpathogenic, vaccine strain) obtained from Colorado Serum Co., Denver, CO and *Bacillus thuringiensis* serovar. *kurstaki* strain 4D7 obtained from Bacillus Genetic Stock Center at The Ohio State University, Columbus, OH. The overnight cultures of *B. anthracis* and *B. thuringiensis* were grown on trypticase soy agar (TSA) plates (Difco, BD, Franklin Lakes, NJ) at 37°C. A few colonies of each strain were resuspended in PBS buffer pH 7.0 and plated on 2×SG[15] sporulation agar plates followed by incubation at 37°C. The spores were collected as soon as the culture reached over 95% of phase bright spores, usually after four days, and resuspended in 2ml of cold sterile milliQ water. The suspension was centrifuged at 4000 ×g for 5 min at 4°C and the resulting spore pellet was resuspended in a new portion of sterile milliQ water. The spores were washed in this way four more times to remove the remaining debris and vegetative cells. Pure spore preparations were stored suspended in sterile milliQ water at 4°C.

3. Results and Discussion

The refractive index of the standard silica particles was investigated as a means of verifying the method using a homogeneous and well characterized sample. Figure 2 shows images of silica particles suspended at the same concentration in liquids of varying refractive index. The refractive index of the particles can easily be visualized as the

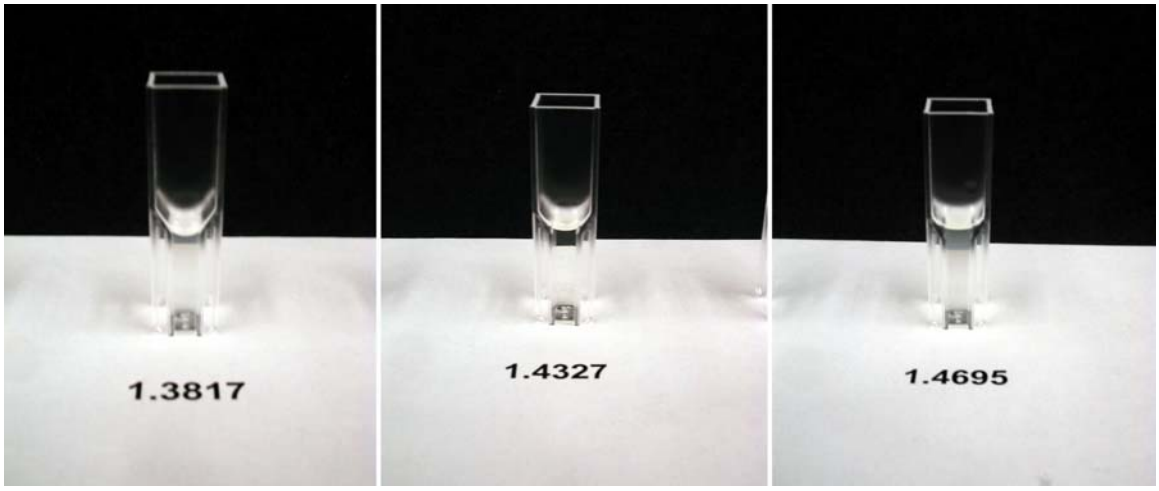

Figure 2. Photographs of 2 μm silica particles suspended in increasing refractive index liquids.

cloudy suspension appears clear when the refractive index of the liquid is close to that of the particles in the middle photo, n = 1.4327. A graph of the more detailed analysis for the silica samples at three different concentrations is given in Figure 3. The optical transmission of the suspensions containing the colloidal silica particles increases with refractive index until there is a close match between the liquid and the particles at 100% transmission. In this case the lowest turbidity of the sample occurs between the values of 1.428 and 1.435.

To obtain an accurate value of refractive index of the silica spheres we measured transmission of the highly concentrated suspensions of silica in the series liquids with refractive indices between 1.426 and 1.434. The results of these measurements are shown on Fig. 4. The peak of the curve fitted to the experimental data (Origin 7, OriginLab Corp, Northampton, MA) was found at the value of 1.4294, which is a close match with the value 1.43 of the refractive index of the particles reported by the manufacturer.

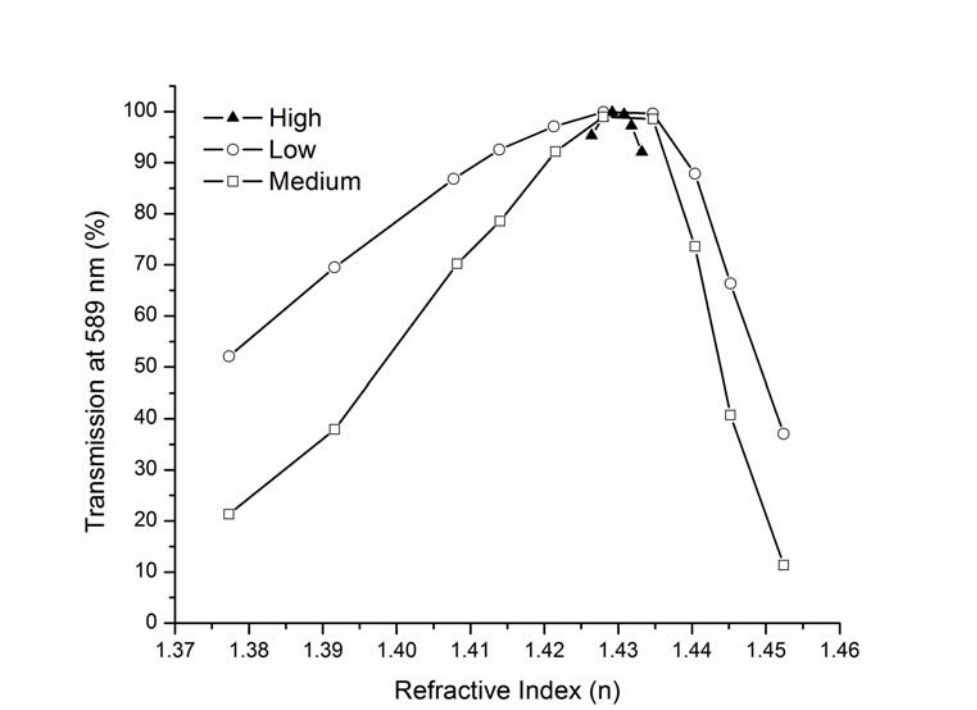

Figure 3. Optical transmission data for three different concentrations of silica spheres (low - 1.7×10^{9}, medium – 4.4×10^{9} and high 1.2×10^{10} particles/mL) suspended in liquids of increasing refractive index

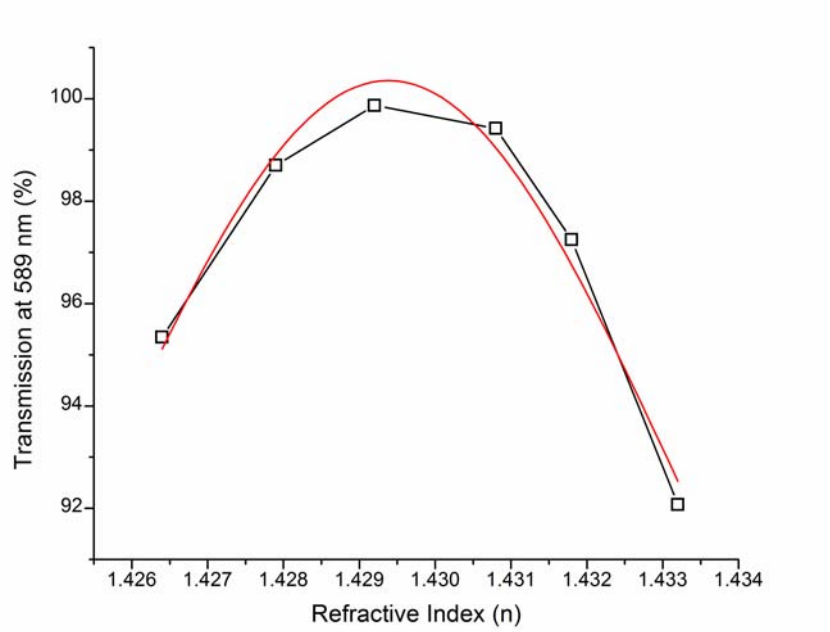

Figure 4. Transmission versus refractive index for highly concentrated silica spheres sample (1.2×10^{10} particles/mL) to accurately determine their refractive index. Curve fitting was used to find the peak value (n=1.4294)

Light microscope images of both species of bacterial samples are given in Figure 5. It is clear from the images that each spore sample has a similar refractive index match with the liquid with refractive index n = 1.53. The instrumental method (discussed below) was required to distinguish the refractive indices of these closely related spore species. The bright refractive nature of both *B. anthracis* and *B. thuringiensis* can be seen in the first immersion liquid in Figure 5, n = 1.337. As the refractive index increases, only certain spores remain visible which is consistent with heterogeneity in the sample. However, at the matching refractive index, very few spores or even shadows can be seen considering that the concentrations are identical. When the refractive index of the immersion liquid becomes larger than that of the spores, they become visible again due to the contrast in the phases.

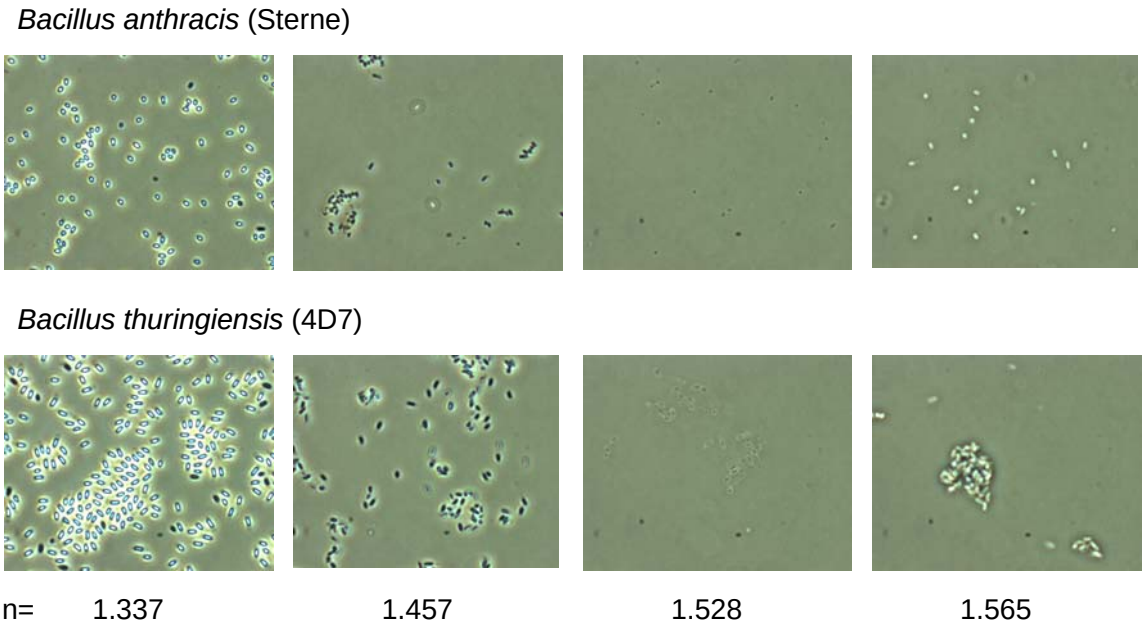

Figure 5. Light microscope images of *B. anthracis* and *B. thuringiensis* spores in liquids of varying refractive index

The instrumental method has been used to measure the refractive index of *B. thuringiensis* and *B. anthracis* spores. Data for *B. thuringiensis* at several concentrations is given in Figure 6. It is clear that higher concentrations must be used in order to properly distinguish the peak region. The low concentration had no curvature to allow discrimination of the peak and the medium and medium-high concentrations had only moderate curvature compared with the high concentration. Figure 7 shows the detailed transmission versus refractive index curves for *B.t.* and *B.a.* spores. Refractive index data

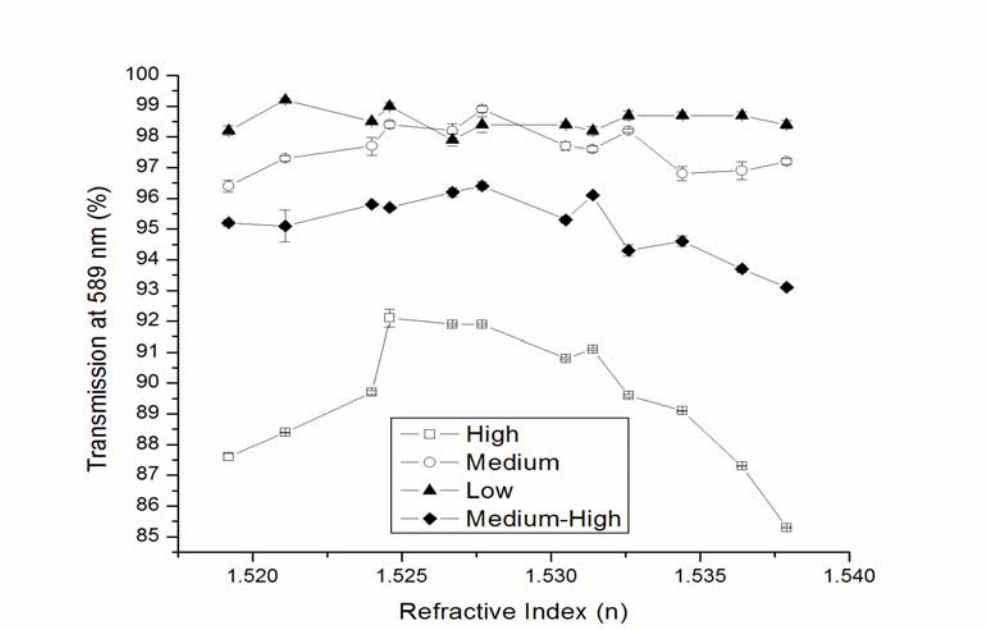

Figure 6. Optical transmission data for *B. thuringiensis* spores in liquids of varying refractive index at four concentrations. Concentrations from low to high were 1.5×10^{8} colony forming units (CFU)/mL, 6.3×10^{8} CFU/mL, 1.4×10^{9} CFU /mL, and 2.9×10^{9} CFU /mL

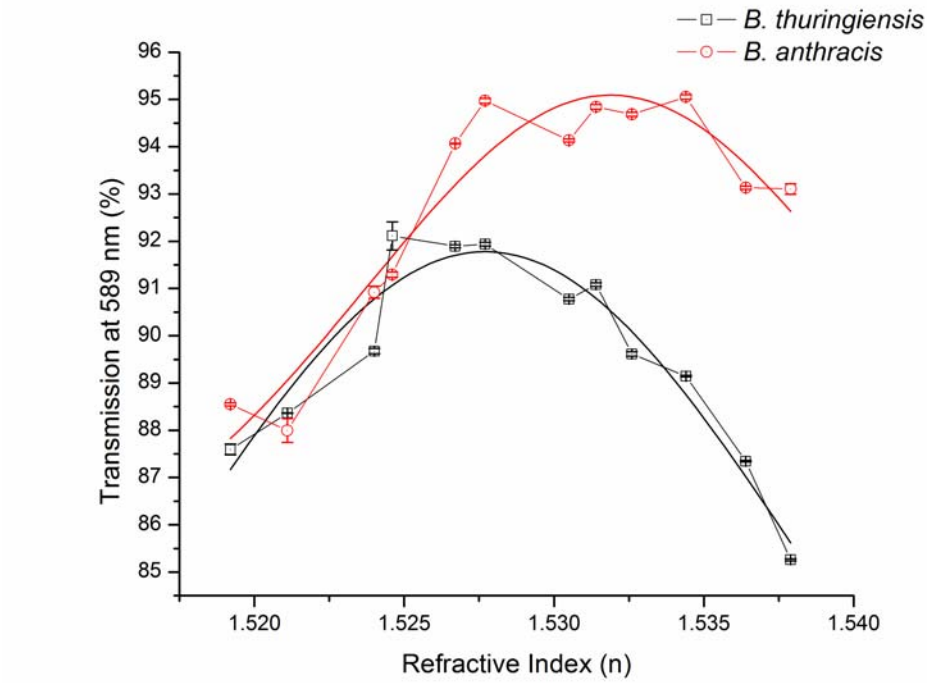

Figure 7. Transmission versus refractive index for *B. thuringiensis*, and *B. anthracis*. Smooth curves were fit using a Gaussian function to determine the peak refractive index values: n = 1.528 for *B.t.* and n = 1.532 for *B.a.*

were obtained from the smooth Gaussian curve fit data for each dataset. The refractive index value for *B.t.* was determined to be n = 1.528, and the refractive index value for *B.a.* was: n = 1.532.

4. Conclusions

A simple and effective methodology for determining the refractive index of colloidal suspensions has been developed. The method involves measuring the optical transmission of colloidal suspensions in liquids of varying refractive index. The maximum transmission point indicates the effective matching point between the colloid and the liquid of known refractive index. The technique has been demonstrated using both inorganic (silica) and biological (bacterial spores) colloidal samples.

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